NUCLEAR MAGNETIC RESONANCE OF WATER IN DEUTERATED CITRUS LEAF TISSUE

By

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Major Department: Horticulture Science

Water in satsuma mandarin citrus leaves was studied using nuclear magnetic resonance (NMR). Differences in the water status of leaves from nonacclimated and acclimated plants were found when observing the resonance signal of H in H_2O and D in D_2O from deuterated leaf disks before, during, and after freezing.

Bandwidth, a measure of transverse relaxation time (T_2) and molecular mobility of the H portion in H_2O, abruptly increased at initial freezing in nonacclimated leaf tissue. Bandwidths in acclimated leaf tissue gradually increased after freezing, suggesting that H_2O might be more structured and more bound to cellular constituents making it less susceptible to crystallization.

The % liquid H_2O remaining after initial freezing and at several subfreezing temperatures was greater in acclimated leaf tissue. When initial freezing occurred, all D resonance signal was lost due to D_2O forming ice, indicating that only the free water in the tissue was subject to replacement by D_2O.

A standard curve for the replacement of tissue water by D_2O was established based on the ratios of H and D NMR spectral areas from prepared H_2O-D_2O mixtures.

Both nonacclimated and acclimated leaves replaced 69% of their total H_2O with D_2O, thereby suggesting this amount to be the free water due to its ready availability for exchange. Therefore, the H resonance signal observed in the deuterated leaf tissue appears to be a measure of bound water.

Initial freezing resulted in a partial loss in the H resonance signal due to bound H_2O forming ice. Acclimated tissues retained a larger % of bound water unfrozen, establishing that a change in H_2O binding to other cellular constituents occurs during acclimation. The prevention of freezing of a larger portion of the bound water provides the greater cold tolerance exhibited by acclimated plants.

Floating leaf disks in D_2O solution was found to be a successful method for deuterating citrus tissue. Plant tissue water can be replaced by D_2O under controlled conditions. However, several deleterious effects of D_2O were observed on plant growth.

INTRODUCTION

Freezing temperature is the single most important factor limiting the distribution and production of plants on the earth's surface (143, 232). Preventive measures to protect plants from freezing include cultural practices (49,114), providing heat to, or maintaining heat within, the plant (3,4,5,12,55,56,57,76,115) and the application of growth-regulating chemicals to induce cold hardiness (27,149,179,185). In addition, research has included selection and breeding of more cold-hardy citrus cultivars (51,52,54,230).

Some plants are extremely resistant to freezing stress and are able to survive the winter to resume growth each spring. Man first thought plants, like animals, possessed a "vital heat" that prevented them from freezing (102). Since that time many attempts have been made to determine why plants develop resistance to cold. Today we know water freezes in hardy plants during the winter, some plants being capable of survival at temperatures as low as -196°C (156). These facts have stimulated considerable interest in the physiological, biochemical and morphological survival mechanisms of plants (61,88,89,90,92,101,166,218).

Plants in the genus Citrus are subtropical and easily damaged or killed by subfreezing temperature. This places definite limits on the areas where citrus can be grown successfully. Although attempts to provide protection to citrus and other marginally hardy crops have been

partially successful, understanding the physiological processes involved in the acclimation to cold would be of great economic value to all plant production.

Changes in water status have been proposed as an important process involved in cold acclimation. Water status has been studied using gravimetric and desiccation techniques. The research in this thesis was designed and executed to determine if nuclear magnetic resonance (NMR) could be used to elucidate further the water status of citrus tissues before and after cold acclimation. Replacement of free water with deuterium oxide (D_2O) was also used to secure additional information. In order to use D_2O it was necessary to develop a method for bringing about absorption and exchange of D_2O with the tissue H_2O in citrus plants.

LITERATURE REVIEW

Acquisition of Cold Hardiness

It is acknowledged that cold hardening in woody plants involves multiple changes in the tissue (126), and that development of cold hardiness is inversely proportional to the growth rate (107). The acquisition of dormancy or lack of shoot, root and cambial growth is the most important factor in the development of cold hardiness (29,79,89,224, 225). Environmental factors that suppress growth, such as low temperature, insufficient moisture, short photoperiod in plants that accumulate starch, and low nitrogen levels, enhance the cold tolerance of most plants. Dormancy and cold hardiness may be separate and parallel processes or merely expressions of a single series of processes (21,31,229).

The term "dormancy" is often used in conjunction with cold hardiness. As used by plant physiologists, dormancy is a period of arrested growth. Rest and quiescence are terms which separate two stages of the dormant period in plants (205). Rest, or winter dormancy, refers to the condition of the plant whereby it requires a cold treatment before it can resume growth even though it is exposed to suitable environmental conditions including temperature and moisture (161). Quiescence is a state in which the growth of the plant is limited only by unfavorable environmental conditions (205,220). Citrus does not acquire winter dormancy and quiescence best describes its reduced growth activity in the winter.

In deciduous plants, the first stage of cold acclimation is induced by short photoperiods (79,158,173,202). In nature this occurs in the fall at the end of the growing season. In hardy woody species growth cessation caused by decreasing photoperiods triggers the onset of acclimation and dormancy. Shortened photoperiods are believed to key entry into the dormant stage rather than to affect the degree of cold hardiness which might be acquired after the plant is dormant (197). Photoperiod has been found to be generally less effective than cool temperature in inducing hardiness.

The second stage of acclimation in deciduous species is induced by low temperature (79,195,202) and is often associated with the first frost in nature. Although the increase in cold resistance during the first stage is relatively minor, a significant degree of hardiness is acquired during exposure to low temperatures (88,89,143,169).

A third stage of acclimation has been defined, but is not normally found in nature (195). Preconditioning with temperatures of -30°C to -50°C has resulted in hardening stems capable of surviving temperatures as low as -196°C (156). However, these stems (79) and buds (147,148) can decrease in cold resistance from -196° to -45° in as little as 6 hours.

There is not enough information available to completely understand cold acclimation in woody plants. However, it is understood that the short-day photoperiod is detected in the leaves (6,48,79,81,86,87), and the message is probably transmitted by way of a translocatable hormone to stop growth and trigger metabolic changes responsible for cold acclimation (7,48,87,204). It has been shown that starch is converted to sugar in plant tissue exposed to low temperatures (81,126,139,143) and

considerable attention has been directed toward explaining the translocatable hardiness factor as a sugar (2,7,10,72,102,104,157,159,177,180). Sergeeva (165) believes monosaccharides are probably the metabolites that promote the synthesis of substances that increase winter hardiness, and that oligosaccharides perform the main protective role.

In citrus, as in other plants, short photoperiods reduce shoot growth (97,145,163,213), but do not induce hardiness. The first step in the acquisition of citrus cold hardiness is cessation of growth (21, 23,28), and 12.8° (55°F) is the generally accepted temperature below which bud and cambial growth cease (20). It is important to note that in hardy, woody species photoperiod acts as an early inductive system to begin acclimation; while in citrus, cool temperature is required for decreased growth and increased cold hardiness (31,142,213,229).

If unseasonably warm weather occurs during the fall and winter, cold hardiness already acquired will be lost (29). If warm temperatures during the winter are prolonged, growth will resume (213). It is recognized that more dormant trees are more cold hardy (21,22,23,25,30,88,89, 90,160,213,224,226,228,232).

The second step in the acquisition of citrus cold hardiness is the hardening process. The degree of hardiness which develops is dependent on the genetic makeup of the plant as well as environmental conditions to which the plant is exposed (89,192,193,194,219,226,229). Varietal differences in cold hardiness, both for seedling and grafted trees, following freezing conditions is well documented (19,26,29,52,54,71,88, 89,104,212,213,214,215,216,217,219,222,228,230,231). Although a number of factors influence citrus cold hardiness, none is more important than

the ambient air temperature prior to the cold experience (31,88,120,169, 213,218,224,229). It has been established that preconditioning plants by exposing them to lower than normal temperatures for a given period of time tends to induce cold hardiness (16,20,21,28,41,43,136,153,174,213,226).

Light and photoperiod are other factors in the cold hardening process (37,128). Short photoperiods reduce photosynthesis and sugar formation. Short day effects are more pronounced in plants grown under hardening temperatures. Plants conditioned under cool temperatures in the absence of light do not accumulate sugars or acquire cold hardiness (136, 219). Many researchers working with citrus have used various hardening regimes to determine the most favorable conditions for hardening (61, 88,91,136,153,174,213,219,226). Citrus plants hardened under cool temperatures below 12.8° and 8-hr days acquire more cold hardiness than plants under long 16-hr days (213). The influence of short days has been linked to less growth activity and more dormant buds and cambium. Although it is acknowledged that 12.8° (55°F) is the generally accepted temperature below which citrus trees increase cold tolerance, not all cultivars cease growth at the same temperature. Young and Peynado (226) using 24 citrus cultivars and 6 related species found the threshold temperature for suppressing growth differed between cultivars when exposed to a 21° day temperature and variable night temperatures. Cold hardiness was acquired by Poncirus trifoliata, 'Nagami' kumquat, False Hybrid Satsuma, and 'Yuma' citrange when exposed to a night temperature threshold of 13.9° to 15.5°. 'Changsha' mandarin and Severina buxifolia had threshold temperature between 10° to 13.9°; 'Redblush' grapefruit, sour orange, citranges and

citrumelus 8.9° to 10°; and limes, lemons and Citrus macrophylla below 8.9°. Varieties which become dormant at higher threshold temperatures are usually more cold hardy when exposed to low temperatures (218,219). It is usually observed that in California, which is characterized by cooler night temperatures, trees become more dormant and are less subject to cold damage than those in either Texas or Florida where night temperatures are usually higher prior to periods of low temperature (28).

Soil temperatures below 12.8° have been reported to be influential in reducing growth and promoting dormancy in citrus (20, 178). Minimum soil temperatures for growth have been reported as 12.8° for orange trees, 10.5° for grapefruit and around 7.2° for lemon (20,68). Root growth of trees usually ceases at soil temperatures below 10.0° (62).

Soil temperatures above 10.0° promote plant growth (137,179); even with air temperatures above 12.8° dormancy is not broken until soil temperatures are also above 12.8° (179).

Many other factors have been shown to contribute to the cold hardiness state of the plant. Light intensity must be supplied at levels which favor photosynthesis and carbohydrate accumulation (37,136,218). Decreased cold hardiness has been shown to occur under conditions when low (90) or high (90,213) light intensities are supplied. Soil moisture influences both time and duration of root and shoot growth, and during extended periods of drought little growth occurs (20). Tree age and size are important for survival during freezing conditions. Unprotected large trees are more tolerant to cold than unprotected small trees (198,227). The difference is related to the heat capacity of the tree and attributed to

the fact that the heat stored in the woody framework and ground beneath the tree is reflected and reradiated with the leafy canopy. Cultural practices involving insect control (80,127,190), fertilizer applications (127,175), irrigation (21,31,82), and crop harvesting (101,127) have all been reported to influence tree damage following exposure to freezing condition. It is understood that a healthy, properly managed tree will acquire maximum cold hardiness under optimum hardening conditions.

Many growth-regulating chemicals have been tested for their effect on dormancy and cold hardiness (21,27,50,59,74,149,185,221). However, none of the growth inhibitors or retardants, including malic hydrazide, have given any real significant cold protection or increased hardiness in citrus (21,27,50,59).

Metabolic Changes Accompanying Acquisition of Cold Hardiness

The metabolic changes associated with cold acclimation in plants have been the subject of much research (36,53,73,93,103,108,109,110, 123,126,143,172,188,207). When plants acquire cold hardiness, by means of natural or artificial conditioning, quantitative and qualitative changes are found in sugars (78,104,116,119,142,218), protein (34,60, 116,118,202), DNA and RNA (38,67,116,117,172), lipids (201,207), protein sulfhydryls (98,106,110,111), bound water (14,104,108), and ascorbic acid (53). Increased cold hardiness has been found to correlate with changes in almost every group of compounds examined.

Studies of metabolic changes in plants have suggested that acclimation is either the result of several independent physiological events whose effects are additive (173), or a sequence of processes that are mutually dependent (204). The hope has been that a diligent search will uncover a single biochemical alteration or sequence of alterations responsible for all plant cold hardiness (126).

The possible role of sugars in the cold acclimation of plants has been the subject of research for many years (107,157,159,177,192). In the fall there is a conversion of starch to sugars in woody plant tissues which are capable of cold acclimation (118,126,140,141,143,157). This increase in reducing and total sugars has also been reported in citrus (89,92,166,218,219,229). It has been suggested that this increase in sugars during the winter, without an accompanying increase in starch may constitute a "cold reaction" in citrus (92). However, Ivanov (88) was unable to find a consistant relationship between cold hardiness and sugar content in citrus leaves. Furthermore, artificially increasing the sugar content in citrus or deciduous (48) plant tissue has never been shown to increase cold hardiness. A decrease in reduced glutathione has been shown to be a better indicator of cold hardiness than sugar content (88, 89,90).

The critical temperature at which starch begins to be converted to soluble carbohydrate in citrus during the winter has been shown to be 12.8° (166). Perhaps the initiation of the starch to sugar conversion is in some way related to the fundamental change in the plants ability to acclimate to cold stress (166). However, the increase in sugars

during acclimation may be coincidental. The low temperature-induced conversion of starch to sugar occurs in many plants such as Irish potato tubers and sugarcane which are incapable of cold acclimation (104).

Decreased total water content and increased bound water have been found to accompany increased cold hardiness in citrus (78,105,186,229). Early investigators unsuccessfully attempted to improve cold hardiness of plants by reducing their water content (15). If, however, the water supply is reduced over a long period of time, definite increases in cold tolerance can be observed (199). These results suggested that it is not the total water content, but only a certain portion of it that is important. It is this portion that has been termed "bound water" and has been the subject of many studies. Gortner and Gortner (65) reported that tender plants did not contain bound water but that hardened plants contained significant amounts. Stark (176) found large amounts of bound water in apple shoots but was unable to distinguish degrees of hardiness. In pine needles (132) a smaller amount of bound water was found in hardened than unhardened needles, and frost-hardy cabbage (108) was found not to contain bound water. This lack of agreement among investigators suggests that the correlation between bound water and hardiness was, and still is, open for investigation.

Bound water as defined by Levitt (102) was of two types, one was water influenced by osmotically active substances and the other water influenced by colloidal substances. The water bound by osmotically active substances is dependent on the solute content. It is the water bound by colloidal substances which is postulated to increase during

acquisition of cold hardiness. It has been suggested (65) that this bound water, due to its orientation around colloidal particles, is removed from the body of the solution and loses its solvent properties. Lauffer (100) found that tobacco mosaic virus (TMV) protein aggregates at room temperature and disaggregates near 0°. Since the process is reversible, water is bound during disaggregation and released upon aggregation. He proposes that water is bound by either the 'iceberg' or hydrogen-bonding mechanisms, since both types of binding are known to respond to temperature changes. At room temperature hydrophobic structuring of water is weak, but the number of ordered water molecules around the nonpolar hydrophobic surface increases as the temperature is lowered (7). On the other hand, hydrogen bonds in macromolecules are broken at low temperature but there exists an increased probability of water-to-water hydrogen bonding (146).

Bound water is less mobile structured water around cell components (134). The binding forces between water and biological macromolecules are either hydrogen bonds between polar groups and water molecules (33, 100,146,162), or hydrophobic bonds between nonpolar residues (7,100,146, 203). Recent research dealing with hydrophobic bonding of water "icebergs" (100) has shown that proteins and nonpolar residues of amino acids like alanine, valine, leucine, cystine, methionine, and phenylalanine provide surfaces which can participate in this type of bonding. Furthermore, we know nucleoproteins and lipoproteins incorporate water molecules which serve a structural and functional role (17,100,124). Nucleic acids (134) and proteins (70,121) are known to bind water amounting to 30% to 50% of their weight.

In citrus, it has been suggested that the increase in sugars during hardening results in more bound water, a greater stability of the tissue water, and less available water for ice crystal formation (220). This hypothesis is supported by the finding that ice nucleation is slower in hardened tissue (229). Further studies have shown that changes occur during hardening which increase membrane tolerance to the presence of ice (218,219).

Substances that inhibit the phase transition of water to ice have been reported (196). This biological antifreeze has been reported to be a glycoprotein in the blood of fish living in the icy waters of the Atlantic Ocean (45). A heat stable protein in spinach leaves protects chloroplast membranes from freezing (72). Polysaccharide polymers extracted from cereal grains interfere with ice crystallization (138).

It is evident that cold acclimation is an active metabolic process, and that physiolgoical and biochemical changes occur during a time when the plant appears to be otherwise dormant.

Methods for Determining Cold Hardiness and Injury

Methods to Determine Hardiness Using Injury to Intact Plants

Visually assessing injury to plants following naturally occurring cold temperature has long been the basis for determining their relative cold tolerance. Many studies of citrus cold hardiness have used this field survival method (24,26,29,54,71,80,101,222,231). Although a general rating of hardiness has been established for most citrus cultivars,

based on the effect of environment on the acquisition of cold hardiness and the genetically controlled ability of the plant to survive freezing, there have been many unexplained exceptions reported. In an effort to establish uniformity, researchers have turned to artificial freezing tests. Good agreement is obtained between the results of controlled freezing tests and field survival observations (25,74,102,174,225,226). Even so, the freeze testing of entire plants requires large equipment and the sacrifice of many plants. Therefore, an indirect method to evaluate cold hardiness without freezing the whole plant is desirable.

Methods to Determine Hardiness Using Plant Parts

In an effort to determine cold hardiness of plants, many attempts have been made to develop a method that will measure the degree of cold tolerance. Many of these methods have serious shortcomings and do not give consistent, reliable determinations of lethal temperatures.

Decreased cambial activity in the stem is associated with dormancy and the ease of separation of the bark from the wood has been used as an index of cold hardiness (25,30,61,82,224,229). However, cambial activity cannot be directly correlated to cold hardiness since it continues throughout the winter and is affected by soil moisture and root activity (25,30,75,224).

The electrolytic conductivity method used for many plants measures the electrical conductivity of the cell sap which has an increased concentration of electrolytes released from the cytoplasm after damage from freezing (39,40,46,96,181,182,183,184,189,208,209). Not all

attempts to use electrical conductance of water leachates to measure damage from cold hardiness have been successful (43,208). This method has been completely unsuccessful in citrus (95,135).

Electrical impedance is often used to measure hardiness and is based on the assumption that intact tender stems offer less resistance to electric current than hardened stems (42,43,44,46,63,64,94,122,206, 208). Comparison studies of the 2 electrolytic methods (9,94) have indicated that electrical conductance of water diffusate from stems and leaves is better for measuring injury, and electrical resistance measured with electrodes placed into the stem is the better method for determining hardiness.

A color test has been developed to distinguish between viable and freeze-injured tissues (150). When placed in tetrazolium red (2,3,5-triphenyltetrazolium hydrochloride) uninjured cells take up the dye but injured cells do not. This test does not indicate which cells will die at a later date but does differentiate between individual dead and living cells. Yelenosky (211) has used tetrazolium reduction of sugar substances diffusing from plant tissues to distinguish between cold-hardened and unhardened citrus plants. The amount of diffusing substances is greater from cold-hardened than unhardened citrus, and the differences are colorimetrically distinguishable. However, this method does not give a measure of damaging or lethal temperatures.

Exotherm analysis has been used for measuring cold hardiness in fruit species. Freezing curves are records of tissue temperature during freezing and indicate points at which substantial water crystallization

occurs due to exotherms that are created when the latent heat of fusion temporarily warms the sample. In plant tissue killed at the moment of freezing (66,151,152), the killing temperature can be predicted simply by recording the temperature of the freezing point.

Exotherm analysis studies have utilized the fact that multiple freezing points are a unique property of living tissue (124,129,130) to test for viability of tissue exposed to freezing temperatures. When hardy stem sections freeze they produce 2 distinct exotherms. If stem sections are killed during freezing, thawed and refrozen, they product only one exotherm, but at a higher temperature. Somewhat similar results are found in citrus (58). However, the initial freezing of a citrus leaf produces a single exotherm and refreezing the same leaf also produces a single exotherm, but at a higher temperature. Freeze disruption of membranes and release of the cell sap accounts for the higher refreezing point. The depressed first freezing point indicates citrus has mechanisms other than increased osmotic concentration for acquiring cold hardiness (58).

Although in some early work with leaf freezing points (LFP) no correlation between LFP and tree hardiness and killing temperature were found (217,229), recent studies have shown LFP to give a valid measure of citrus cold hardiness and to correlate with lethal temperatures (58, 85,91). The leaf freezing curve pattern of 6 different citrus cultivars was studied (223) and found similar for all.

Investigators (58,91) have used LFP to evaluate cold hardiness of seedling citrus plants and have found good correlation between LFP and

low temperature damage. Hutcheson and Wiltbank (85) using LFP to measure the cold hardiness of container-grown budded citrus plants also found a direct correlation between LFP and killing temperature as determined by actual freezing tests. This relationship holds for additional citrus varieties and orchard trees (83,84). Additional studies (120,155) using LFP found them to be a reliable measure of citrus cold hardiness. The LFP method has been adapted for portable use (210) and field tested using distant locations (155). The LFP method has thus been shown to be a valid means for determining the cold hardiness of several citrus cultivars.

Nuclear Magnetic Resonance: A New Tool for Studying Plant Water

Basic Principles of Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) provides a useful technique for studying water in plants. Until just recently (13) NMR had not been used to study the status of cellular water in relation to freezing injury and cold hardiness in plants. NMR can be a convenient tool for studying water in plants because it is nondestructive, and provides structural as well as dynamic information. NMR yields information by which the amount as well as the state of unfrozen water in plant tissue at low temperatures can be determined.

The following discussion of the principles of NMR has been compiled from several textbooks and other sources (1,11,112,144,154,171). The first experimental realizations of NMR occurred in late 1945. It was

not until 1951 that the "resolution" of the spectrum of ethanol into 3 peaks (CH_3, CH_2, OH) was reported. In 1953 the first commercial "high resolution" NMR spectrometer was sold. Not only has NMR become a scientific discipline, but it has become a routine analytical tool in many fields of research, especially organic chemistry.

An NMR spectrometer consists basically of a magnet, radio-frequency (RF) transmitter or oscillator, and a suitable RF detector. When a sample consisting of atoms having nuclei with certain magnetic properties is placed in the magnet pole gap and subjected to the RF field, absorption of RF energy (resonance) occurs at particular combinations of the oscillator frequency and the magnetic field strength, and an RF signal is picked up by the detector.

The oscillator coil is oriented with its axis perpendicular to the principal magnetic field. The receiver coil is tuned to the oscillator frequency and is oriented with its axis perpendicular to both the direction of the principal magnetic field and the axis of the oscillator coil. This arrangement is to minimize overloading the sensitive receiver which would result from direct coupling between the oscillator and receiver coils. Thus the resonance signal arises from indirect coupling between the oscillator and receiver coils produced by the sample.

It is helpful to use an oversimplification of the real state of affairs in explaining the magnetic properties of nuclei and how a nuclear resonance signal can arise.

Certain nuclei behave as though they are nonspinning spherical bodies with the nuclear charge distributed evenly over their surfaces.

This type of nucleus does not have a magnet moment because there is no circulation of the nuclear charge. These nuclei are said to have their nuclear spin value equal to 0 and, not having a magnetic moment, they can give no nuclear resonance signal. Many important nuclei, ^{12}C and ^{16}O, are of this type, in fact this is true of all nuclei whose mass numbers A and charges Z are both even; A = Z + N. When it is necessary to observe a carbon resonance signal the isotope ^{13}C can be used. The natural abundance of ^{13}C is only 1.1%.

A number of nuclei have nuclear spin values of ½. This means that they act as if they were spherical bodies possessing uniform charge distributions but spinning like tops. A spinning nucleus has circulating charge, and this generates a magnetic field so that a nuclear magnetic moment results. Nuclei with spin ½ include ^{1}H, ^{11}B, ^{13}C, ^{15}N, ^{19}F, and ^{31}P. Such nuclei are particularly favorable for NMR experiments.

A very large number of magnetic nuclei act as though they are spinning bodies with nonspherical charge distributions and are assigned spin values of unity or larger multiples of ½.

In the magnetic field an isolated nucleus of spin ½ has 2 available energy states, corresponding to alignment with and against the field. A slightly larger number of nuclei will align with the field because of the lower energy state. Absorption of energy and resonance occur when the correct combination of magnetic field and radio-frequency are supplied. This irradiation with electromagnetic energy at a specific radio-frequency induces transitions between the 2 energy levels. The difference between the upper and lower states is very small, only a few

millicalories per mole. Thus at room temperature only a very few excess nuclei are in the lower energy state, according to Boltzmann distribution law. It is this small excess that gives rise to an observable NMR signal. For hydrogen, the population distribution is 0.99999/1.00001 at 10,000 gauss and at room temperature. This small ratio demands sensitive detection techniques.

Absorption of energy results in the excitation of the nucleus to a higher energy state. A net initial absorption of energy is observed until equal populations are attained, whereupon further absorption will cease due to saturation. The rate at which saturation is attained depends on the intensity, or amplitude of the exciting radiation and on the rate at which spin excited nuclei return to the spin ground state, termed relaxation. In an NMR experiment, it is most desirable to maintain sustained absorption of the radio-frequency energy and to avoid saturation.

Relaxation processes can be divided into 2 categories, spin-lattice and spin-spin relaxation. Both the interactions between nuclear spin systems and the lattice as well as the effect of interactions between spins themselves must be considered. Spin-lattice, or longitudinal relaxation involves the conversion of the spin energy into thermal energy. The lattice is defined as the aggregate of atoms or molecules under study, whether it be solid, liquid, or gas. The lifetime of an ensemble of identical nuclei not being irradiated in either the upper or lower energy state is described by T_1, the spin-lattice relaxation time. When the nuclei are irradiated, the Boltzmann distribution is disturbed.

With time there is an increase in the sample magnetization along the field axis as more of the nuclei drop into the lower energy state with magnet quantum number +½. T_1 thus defines the time required for the nuclear spins to exponentially return toward Boltzmann population.

Spin-spin or transverse relaxation involves a transfer of spin energy of one nucleus to a neighboring nucleus, the rate of transfer being denoted as the relaxation time T_2. Each nuclear magnet finds itself not only in the applied steady magnetic field, but also in a small local magnetic field produced by the neighboring nuclear magnets. It is convenient to describe the spin-spin interaction T_2 as the lifetime or phase-memory time of a nuclear spin state, where $T_2 \approx 10^{-4}$ sec. Thus the precession of the spins of 2 nuclei will initially be in phase and then with time will be out of phase. If the nuclei lose phase coherence, the macroscopic component of magnetization in the XY plane goes to 0. T_2 is the time constant for the kinetically first-order decay of X, Y magnetization.

Generally, an increase in the viscosity decreases T_1. Similarly, the presence of paramagnetic materials or atoms possessing an electric quadrupole moment causes T_1 to decrease. Both T_1 and T_2 affect the width of the absorption line, the width increasing as T_1 and T_2 decreases. Further, the area under the absorption peak is directly proportional to the number of atoms giving rise to the signal.

There is little doubt that biological systems contain liquid water at subfreezing temperatures, and that the amount of this bound water decreases with decreasing temperature (102,191). The suggestion that

bound water may play a role in cold hardiness has led to much confusion (102,105). This has been due to erroneous methods of measurement and to what Levitt (102,105) considers confusion between osmotically bound and colloidally bound water, in spite of the fact that no difference exists in the nature of the water which is bound. The sole purpose of distinguishing between the 2 is in order to discover which cell components are responsible for the effect (105). Chandler (14) outlined the most frequently used methods by early investigators based upon the colligative properties of a solution, i.e., vapor pressure lowering, freezing point depression, and osmotic relationships. Current usage in cryobiology loosely defines bound water as that which does not freeze (131). Mazur (125) concluded that the unfrozen water in cells is water bound to cellular solids. Other studies have compared the quantity of water removed in 2 consecutive stages of drying (105,229), or by a mathematical formula proposed by a Soviet investigator comparing tissue fresh weight and liquid-infiltrated weight to dry weight (164).

One fact that is clear from the bound water controversy is that the quantity of bound water can be defined to the satisfaction of all investigators only on the basis of the method used to measure it. Since so many different methods have been used, it is obvious why so many definitions have emerged. The fraction of the total water that is taken as bound water will vary with the method used.

Some progress in measurement of bound water appears to be possible utilizing NMR spectroscopy. NMR measures the total number of hydrogen atoms present. Hydrogen atoms of solids, because their molecular motion is restricted, exhibit broad resonance lines while hydrogen atoms of

liquids give sharp peaks. Because of this disparity, it is possible to consider only the signal coming from liquid water and ignore that from solids. NMR line widths for solids are about 30,000 Hz or broader, line widths for ice fall in the 100,000 Hz range, while line widths for aqueous phases in tissues range from several Hz to about 8,000 Hz. NMR has been used to quantitatively measure the liquid water in frozen wheat flour dough (18,133,187,191). High resolution NMR spectra analysis shows that bound water is not "icelike" in any literal sense, although it is less mobile than liquid water at the same temperature.

Numerous studies of the water of living cells using NMR techniques have appeared in the literature, in which the spin-spin relaxation time T_2 and the spin-lattice relaxation time T_1 of the hydrogen nuclei have been measured. Early NMR studies were for the quantitative determination of water content (167,168). More recent studies using NMR have found it to be a powerful tool for the study of the structure of cell water because NMR spectra and relaxation times are influenced by the chemical and physical environment surrounding the individual proton. Broadening of the proton NMR spectra of water has been observed in biological systems. All of these studies show that both the longitudinal or spin-lattice relaxation time T_1 and the transverse or spin-spin relaxation time T_2 are reduced in living cells compared to their values in pure water. It is understood that broadening bandwidth due to shorter relaxation times implies an ordering of the major portion of cell water.

Deuterium Oxide in Biological Systems

Important information can be obtained from NMR studies by substituting the deuterium nucleus for that of hydrogen. This is due to the greater sensitivity of D relaxation times to changes in molecular mobility, and to the lack of interference of the D resonance by resonance of H in cellular components, such as might occur from protons in animal tissue proteins (69). Furthermore, because of its electric quadrupole moment, the deuterium nucleus yields information about microscopic electric fields in tissue water. In contrast, NMR study of hydrogen only yields information on microscopic magnetic fields because hydrogen lacks a quadrupole moment (33). This is a major advantage of D use.

Information about deuterium up to 30%-35% of the body fluids in mammals from drinking water is available. However, information about the effects of D_2O on plant growth is limited. Since the first experiments on the inhibition of the development of tobacco seeds (113), seed germination studies have remained of primary interest in studying the deleterious effect of D_2O on plant growth. Although complete deuteration of higher plants has not been accomplished, previous experimentation has shown a reduction in seedling growth proportional to the D_2O content, and a complete lack of germination in 99.8% D_2O (8,35,47,170,200). Cope (32) and others have shown D_2O inhibits cell division. In an experiment with higher plants, duckweed, he found intracellular water is consistently much lower, 10% to 15%, in deuterium than is the medium. He suggests there is operative in these plants a system which cannot only distinguish between the 2 types of atoms but can selectively retain 1, either by uptake or transportation. Kutyurin (99) found that when Elodea canadensis was soaked in H_2O-D_2O mixtures it accumulated H_2O preferentially.

MATERIALS AND METHODS

Nuclear magnetic resonance (NMR) was used to investigate the water status in citrus leaves before and after cold acclimation. Further information was obtained by observing the deuterium signal when the free water was allowed to exchange with deuterium oxide (D_2O). Changes in water status were then compared to changes in cold hardiness.

Plant Materials

From a group of uniform 18-month-old seedlings of 'Owari' satsuma (Citrus unshiu Marcovitch) mandarin, 24 seedlings were selected and divided into 12 replications of 2 plants each. The seedlings were grown 1 per #404-700 (46 oz) can container in a greenhouse with natural daylight. Nutritional requirements were supplied by addition of Ortho-Gro liquid plant food 12-6-6 with Fe and Zn every 4 weeks.

Treatment of Plant Materials, Sample Collection and Analysis

At the beginning of the experiment NMR spectra were recorded at several temperatures and cold hardiness determined by the leaf freezing point (LFP) method for each of the 12 replications of greenhouse-grown plants which were in a nonacclimated state. Eight replications were then

subjected to chamber-controlled cold acclimating temperature conditions, and 4 replications were returned to the greenhouse. LFPs were determined at 2-week intervals on all replications. Following 7 weeks of conditioning the NMR water spectra and LFP were again determined. Furthermore, tissue samples were collected and deuterated both before and after cold acclimation. NMR H in H_2O and D in D_2O were determined for these samples.

Cold Hardiness Determinations

Plant cold hardiness tests were conducted at the beginning of the experiment, at 2-week intervals during acclimation, and at the termination of the experiment on all replications. The leaf-freezing point (LFP) method of Hutcheson and Wiltbank (84,85) was used. Three fully expanded mature leaves from each plant were collected and their LFP determined. A mean LFP per plant was calculated and the mean of 2 trees of each replication were averaged to give the mean leaf-freezing point (MLFP) for each replication. LFP values were determined in the lab using a portable leaf-freezing unit similar to that described by Rouse and Wiltbank (155,210). Each leaf was folded around and secured with a paper-clip to a thermistor (Atkins 10993-5 bare bead) attached to the end of a 38 cm by 1 cm diam wooden dowel. The mounted leaf was then lowered through a port in a specially designed and insulated lid of a Norcold MRTF-614 portable marine freezer. Freezer temperature was maintained at $-17.8°C \pm 1°$.

The thermistor, which serves as 1 resistor of a Wheatstone bridge arrangement, was connected into an Atkins 24108-30 temperature indicator

and through an Atkins 21092 recording scale-expander to a Heath/Schlumburger SR-255B potentiometric recorder.

Cold Acclimation

The plants to be cold acclimated were transported to the U.S. Horticultural Field Station of the United States Department of Agriculture in Orlando, Florida, where they were acclimated in the chamber described by Young and Peynado (229). These plants were exposed to day/night temperatures of 21.1°/10° for 2 weeks, followed by 2 weeks at 15.6°/4.5° followed by 3 weeks at 10°/-1.1°. Chamber temperature was controlled accurately to within $\pm$ 0.5°. Light readings taken at plant height were 240 microeinsteins[1] or 1200 ft-c, supplied from a combination of fluorescent and incandescent bulbs.

Deuteration of Plant Samples

Leaf disks 10.5 mm in diam and suitable for use in the 12 mm O.D. glass sample tube of the NMR spectrometer were cut using a standard cork bore tool. A 4.0 mm hole was cut in the center of each leaf disk in order to accommodate a specially made glass tube containing TMS (Tetramethylsilane) as a proton reference when observing the deuterium resonance signal. Four to 5 mature leaves from each replication were used

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[1] $E = h\nu$, hence 1 microeinstein = approximately 0.533 cal. at 400-700 nm (161).

to prepare 70 leaf disks for each sample. Caution was taken to avoid including the leaf midrib in an effort to maintain uniformity and to aid in packing the NMR tube. Each sample of 70 leaf disks were floated in 2 petri dishes (8 cm diam) each of which contained 10 ml of 99.7% pure D_2O. The petri dishes containing leaf disks were placed under a light bank of 4 fluorescent bulbs (40 watt, cool white) for 3 hr. The leaf disks were then removed from the D_2O and placed on a paper towel until the surfaces were air dry (approximately 25 min).

After air drying, the leaf disk were stacked on the 4 mm O.D. TMS tube. The tube plus leaf disks were weighed and placed inside the 12 mm NMR sample tube. A small piece of cork placed in the curved bottom of the NMR sample tube provided a flat surface on which the leaf disk could be vertically stacked. A specially cut spongy piece of 12-mm-diam, styrofoam-like insulation material with 4 mm center hole was position above the leaf disk to maintain packing. The prepared sample tube was inserted into the instrument probe, and the desired temperature was established. Leaves from nonacclimated and acclimated plants were prepared in the same manner. Fresh wt of the leaf disks were accurately determined by subtracting the wt of the glass tube from the total wt of the tube plus leaf disks.

NMR Measurements

At selected temperatures proton spectra of H and D were recorded until the H or D signal disappeared indicating all H and D nuclei were immobilized due to freezing. To establish reference values for unfrozen

tissue, spectra were recorded at temperatures above initial freezing. These temperatures were 10° and 0°. The selection of the initial freezing temperaturesing temperatures (the temperature corresponding to the greatest loss of NMR signal area) was determined by the average LFP and was the temperature at which the sample was expected to begin freezing. For the nonacclimated plants at the beginning of the experiment this temperature was -5°. For the acclimated plants this temperature was -7.8°. After the initial freezing, the subfreezing temperatures used for the nonacclimated plants were -10°, -15°, -25°, -50°. In determining the temperatures for the acclimated plants the scale was shifted by the difference in average LFP. However, freezing in the NMR did not begin until -10°. Therefore temperatures of -10°, -15°, -20°, -25°, -30°, and -50° were used.

NMR measurements were made on spinning and stationary samples and saturation was avoided. When observing the proton resonance signal no difference was noted between a spinning and nonspinning signal. Therefore these spectra were recorded spinning. Although spinning caused no change in the deuterium spectra, the H signal of the reference TMS gave a sharper peak when not spinning. Since the ability to lock on the TMS was dependent on a sharp signal, the sample was stationary when observing deuterium resonance.

NMR measurements were made on a conventional Varian XL-100 NMR spectrometer operated continuous-wave at 100 MHz using supplementary circuitry for observation of the deuterium signal on the X-Y recorder. This instrument was made available by the Department of Chemistry, University of Florida. Temperature in the probe chamber was established

and maintained using liquid N_2 as a coolant and an electronic temperature controller connected to a remote sensor in the probe chamber. Because of the importance of accurate temperature in this experiment, the chamber temperature was verified using a supplementary thermistor-equipped electronic temperature indicator and by means of a standard alcohol calibration curve.

Instrument settings for the spectrometer were as follows. When observing the H signal of H_2O the lock channel was on external H_2O with an RF field of 60 dB. The RF observe channel for H was set at 50 dB with a sweep offset of 82,201 Hz. Sweep width and time were 2,500 Hz and 250 sec respectively. When observing D the lock and observe channels were interchanged, using the lock signal circuit as the observe circuit, and vice versa, in order to use TMS as a lock signal in place of the instrument lock on external H_2O. When observing D in this manner the lock signal was H of TMS with the RF field at 45 dB. The observe RF field was 80 dB and the sweep offset was 84,501. Sweep width and sweep time were the same as for H of H_2O.

Resonance spectra of individual samples requiring approximately 5 hr per sample were determined at selected decreasing temperatures without repositioning the sample in the spectrometer. Line widths, a measure of molecular mobility relating how tightly the water is held, were expressed as widths at half the maximum height of the absorption line. Area under the absorption line was also computed. Changes in the area, representing changes in liquid water, were plotted as area versus temperature.

Analysis of NMR Data

Development of Standard Curves

In order to correct for temperature and other inherent effects which cause an increase in signal with decreasing temperature of a fixed amount of H source, the signal from ethylene glycol was recorded at the temperatures used in this experiment. A line of best fit was established and used as a temperature correction curve for the tissue samples.

The amount of sample deuteration was determined using prepared standards of H_2O-D_2O mixtures. By computing the ratio of H to D in the prepared mixtures, a known computed ratio was determined. The NMR signal of H in H_2O and D in D_2O in these prepared mixtures was observed on the NMR spectrometer and an observed ratio was computed based on the areas under the absorption band of H and D. The known computed ratio from the prepared mixtures was plotted against the observed ratio of areas under the absorption band from the NMR resonance signals, and a line of best fit was established. A ratio of areas under the absorption bands for H and D in a plant sample could then be compared to this standard curve and the degree of replacement of H_2O by D_2O determined.

Comparisons of Data from NMR Spectra

Two types of measurements were considered. These were line widths (bandwidth = $\Delta\nu_{\frac{1}{2}}$) expressed as widths a half the maximum height of the absorption peak, and area under the absorption peak. The area under the absorption band is proportional to the liquid water and the bandwidth provides a measure of how tightly bound the liquid water is; the wider

the band the more tightly bound the water. The major strength of continuous wave NMR is for determining the amount of liquid water in frozen tissue.

An experimental design was established that would facilitate comparisons between NMR spectra within each sample at different temperatures as well as between different samples at a given temperature. Primary consideration was given to comparison of the H_2O spectra of plants before and after cold acclimation. Using the spectra area at 0° for each sample as a reference point and correcting to 0° as representing 100% of the proton water in the sample, the % water remaining liquid was computed for each of the subfreezing temperatures at which spectra were recorded. Not only were these values for nonacclimated tissues compared to the values for acclimated tissues at a given temperature, but comparisons were made at temperatures which were the same number of °C below the freezing point for each respective group of plants. This was made possible by using LFP values and shifting the temperature scale at which NMR spectra were made by the same number of °C that LFP changed during acclimation.

The same consideration was given to the deuterium spectra which were recorded as long as sufficient D_2O remained unfrozen to give an observable signal. Consideration was also given to the replacement of H_2O by D_2O. Evaluation was made of changes in the amount of D_2O in the sample as temperature decreased as well as the amount of D_2O remaining after freezing.

Line widths were evaluated with respect to molecular restriction and molecular binding. H and D spectra bandwidths for nonacclimated and

acclimated samples were compared at each temperature that they were recorded.

Preliminary Experimentation to Establish a Method for Deuterating Citrus Leaf Tissue

Several methods were tested in an attempt to introduce D_2O into citrus leaves for exchange with the tissue H_2O. These include using artificial light and positioning excised leaves or terminal stems in a beaker with petioles or stems immersed in 99.7% D_2O. Under the same environmental conditions, experiments were performed using intact 15-month-old seedlings repotted from soil into glass beads watered with 99.7% D_2O. Similar plants repotted from soil were grown in solution culture using containers made from black-painted Erlymeyer flasks, given aeration and Hoagland nutrient solution (77) made with varying concentrations of D_2O. These plants were grown in H_2O-D_2O solutions of 33%, 50%, 70%, and 99.7% D_2O. A control in 100% H_2O was maintained under the same conditions of 12-hr light and dark periods. Experiments using leaf disks floated on 99.7% D_2O in petri dishes under artificial light were also run. Experiments using all of the above-mentioned techniques were performed in an effort to yield the most satisfactory method for replacing H_2O in citrus leaves with D_2O.

Each method for deuterating citrus leaf tissue was tested for deuterium content by observing the NMR resonance signal of H and D of H_2O and D_2O respectively. These spectra were recorded from leaves removed from plants after 3, 4, 7, 14, and 21 days of D_2O treatment. Samples, from all treatments except leaf disks, consisted of 4 leaves from

each plant tightly wrapped around a 5 mm O.D. NMR sample tube with the leaf midrib horizontal to the plane of the tube. The 5 mm tube with leaves was then positioned inside a 12 mm O.D. sample tube.

Additional measurements were made on these plants. Transpiration measurements were taken using a Lambda Instrument Corporation model LI-60 diffusive resistance meter. Transpiration measurements provide a measure of water movement through the leaves and an indication of water uptake. Data of fresh wt and dry wt were recorded on all samples before and after NMR analysis. A record of the extent to which plants recovered after being repotted into soil following their exposure to solution culture in D_2O was also kept.

RESULTS AND DISCUSSION

Changes in Plant Cold Hardiness

LFPs were determined for all 12 pairs of plaints during acclimation at 2-week intervals on dates corresponding to the completion of each temperature cycle in the acclimation chamber (Table 1). The LFPs taken after 2 weeks at temperatures of 21.1°/10.0°, and again after 2 weeks of 15.6°/4.5° showed the greenhouse plants acclimating at the same rate as those in the acclimation chamber. This was attributed to low night temperatures which occurred with increasing frequency and ranged as low as 7° outside the greenhouse during the first 4 weeks of acclimation. These temperatures occurred due to a loss of heating in the greehouse which allowed inside temperatures to approximate outside ambient temperatures. The occurrence of these temperatures was significant since 12.8° has been considered the threshold temperature for induction of dormancy and cold hardiness in citrus (21,23,28). Further, cold-hardy cultivars such as satsuma mandarin have been shown to become dormant at threshold temperatures of 13.9° to 15.6° (219).

During the 5th week of acclimation the heating system was repaired to working service. At the end of the normal 6-week acclimating scheme the LFPs of chamber acclimated plants was approximately -8° and those plants in the greenhouse were -6.6°. The plants were allowed to remain 1 additional week at the then current conditions of 10.0°/-1.1° for

Table 1. Leaf-freezing points (LFP) of satsuma mandarin leaves during cold acclimation in a temp controlled chamber and naturally in a greenhouse.

Treatment	Leaf-freezing point[z] (°C)				
	week				
	0	2	4	6	7
Chamber	-5.1	-6.7	-7.0	-8.0	-7.8
Greenhouse	-4.9	-6.4	-7.0	-6.6	-6.8

[z]L.S.D. 5% = 1.2

chamber acclimating plants, and heated greenhouse for the greenhouse plants. At the end of a total of 7 weeks acclimation the chamber acclimated plants had an average LFP of -7.8° and the greenhouse plants -6.8°. Air temperature data taken after the acclimation period have shown that with the heating system operating, air temperatures inside the greenhouse are 10° to 15° when outside air temperatures reach 0° to 10°. Night air temperatures outside during the final 3 weeks of the acclimation scheme were regularly o° to 15°. Further, the occurrence of dormancy-inducing temperatures in the greenhouse during this period was indicated by the fact that none of the many citrus cultivars and species in the greenhouse initiated new growth during this period.

It was concluded from the evidence of LFP and air temperature data during the period of acclimation that both the greenhouse plants and plants placed in the climate-controlled chamber had acclimated. Both groups of plants were considered as plants acclimated to the same degree when the NMR data were recorded in December.

NMR Proton Measurements

Standard Curve for Temperature Correction of NMR H_2O Spectra

When recording NMR spectra of H in H_2O at several different temperatures, the area under the absorption band must be corrected for sample temperature. This correction was made by recording the spectrum of ethylene qlycol at the temperatures to be used for citrus leaf samples and then plotting a graph of temperature vs change in area (Fig. 1). The areas were standardized to 0°. It was estimated that by using strictly

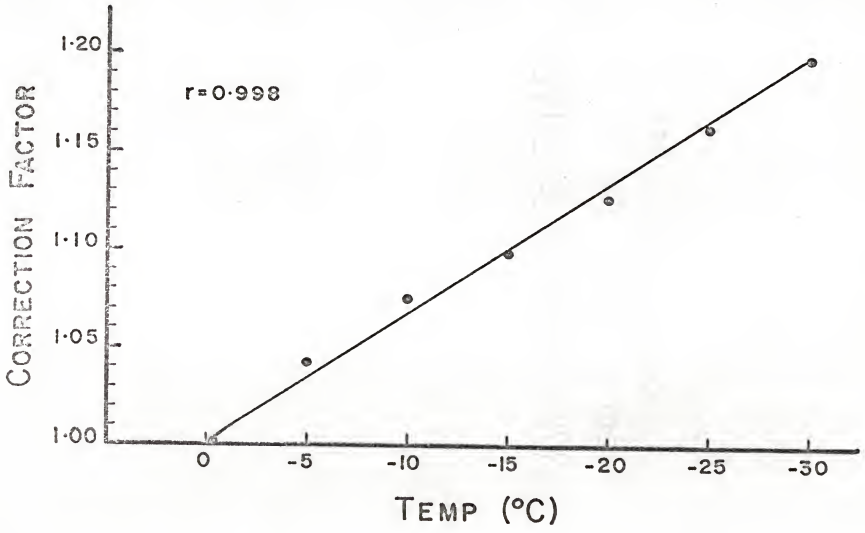

Fig. 1. Standard curve using ethylene glycol to correct for area change of the H O spectra due to temp. Correction factor = A_t/A_o; where A_t = area at some temp., A_o = area at 0°C.

a mathematical correction for the change in Boltzmann distribution of nuclei with temperature, only about 50% of the necessary correction was obtained. The failure to account for the additional correction must be attributed to instrument and other inherent error. The use of a standard like ethylene glycol provided an accurate means to measure the effect of temperature and determine the necessary correction without accounting for the source of error.

The Effect of Acclimation and Freezing on H_2O Protons

The NMR resonance signal of H in H_2O from deuterated leaf samples of citrus was recorded before and after cold acclimation. The area under the absorption band for each sample, which represents the % H_2O remaining liquid at that temperature as compared to the total proton H_2O at 0° in the sample, was recorded at several decreasing temperatures (Table 2). Typical NMR spectra of unfrozen deuterated and nondeuterated leaves are leaves in Fig. 2. By definition in this research, the H resonance signal from deuterated tissue represents bound water, the free water having been replaced by exchange with D_2O. Means of the 12 samples for each temperature are represented graphically as the % liquid H_2O remaining at the selected temperatures (Fig. 3A), and as the % liquid H_2O remaining at temperatures equi-distant from the initial freezing point (Fig. 3B). The initial freezing point is the temperature at which the greatest loss of NMR signal occurred due to the freezing of H_2O into ice. This occurred at -5° in nonacclimated leaf tissue and at -10° in acclimated tissue. The temperatures of these initial freezing points as shown by a decrease in the resonance signal are in close agreement with the LFP values of this tissue (Table 1).

Table 2. Area of the NMR absorption band of H in H_2O as % of initial area of unfrozen, deuterated satsuma mandarin leaf tissue at 0°C.

Temp	Area of absorption band												
	Sample[y]												Mean[z]
	1	2	3	4	5	6	7	8	9	10	11	12	
Nonacclimated													
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0a
-2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0a
-4	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0a
-5	20.4	17.6	21.1	23.1	22.9	27.4	27.8	27.6	28.7	24.2	28.0	35.4	25.4b
-10	17.1	14.6	19.0	14.6	16.0	13.0	18.3	20.0	17.8	13.0	19.4	21.6	17.0c
-15	11.5	11.2	13.0	10.8	9.3	8.1	12.1	13.0	11.7	10.1	13.3	15.2	11.6d
-25	6.8	8.0	5.9	7.0	3.5	3.7	8.2	6.7	7.6	5.7	8.9	8.2	6.7e
-50	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Acclimated													
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0a
-5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0a
-7	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0a
-9	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0a
-10	40.4	41.4	43.0	43.6	36.5	42.1	38.3	41.8	38.3	35.0	41.1	38.3	40.0f
-15	27.8	28.7	28.2	28.8	28.0	28.3	27.6	28.9	28.7	27.1	30.2	25.8	28.2g
-20	20.7	21.8	21.4	22.9	17.6	20.3	19.0	19.4	17.7	18.1	22.9	21.1	20.2h
-25	15.9	13.7	15.8	15.5	12.6	13.5	12.7	16.2	11.4	13.4	18.2	18.3	14.8i
-30	12.0	9.0	11.0	7.5	9.6	11.6	9.2	13.6	8.7	12.9	14.4	15.7	11.3j
-50	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[y]Each sample included leaves from 2 experimental plants.

[z]Means of the 12 samples followed by different letters differ at the 5% level as determined by Duncan's multiple range test.

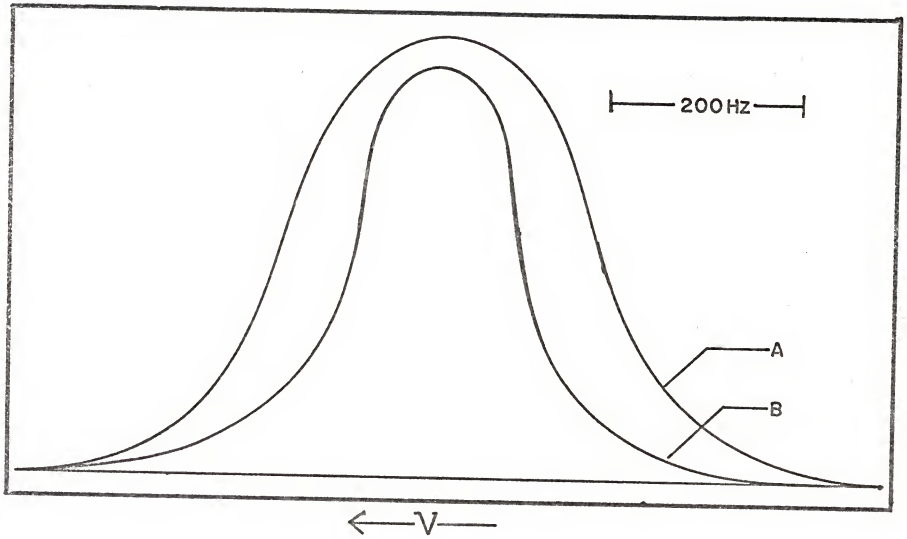

Fig. 2. 100 MHz NMR H spectra of unfrozen satsuma leaf disks. A) Spectrum from nondeuterated leaves. B) Spectrum from deuterated leaves. V is the frequency in Hz.

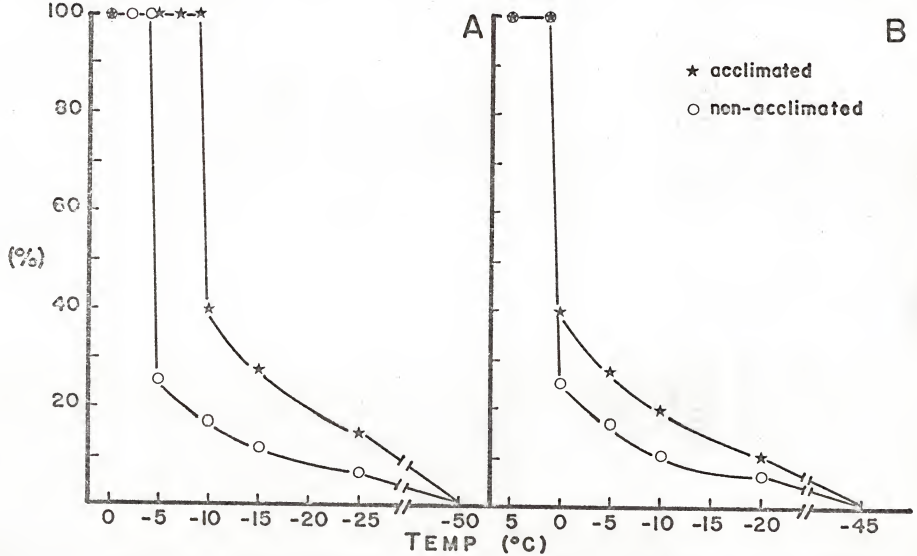

Fig. 3. NMR H spectra areas representing unfrozen H_2O in deuterated leaf disks from nonacclimated and acclimated satsuma plants at: A) actual temp, and B) at temp equi-distant from the initial freezing point. Area is expressed as a % of the spectrum area of 0°C.

At temperatures above the freezing point of the tissue H_2O the NMR spectrum of nonacclimated deuterated citrus leaves had a single H_2O absorption peak with a bandwidth of 180 Hz (Fig. 4). In contrast, acclimated leaves displayed a slightly broader bandwidth of 225 Hz (Fig. 4). These bandwidths are broader than that of H_2O in muscle which is 12-17 Hz (33), and broader than that of pure liquid H_2O which displays a bandwidth of about 1 Hz. However, these deuterated citrus leaves displayed a narrower H_2O bandwidth than nondeuterated citrus leaves (Fig. 2) tested during preliminary studies. Bandwidths were about 200 Hz for deuterated leaves and 400 to 500 Hz for nondeuterated leaves (Fig. 2).

The bandwidth for nonacclimated leaves abruptly increases when freezing begins at -5° (Fig. 4). The spectral bandwidth then broadens progressively more but at a lower rate as the temperature is lowered. In contract, the bandwidth of acclimated leaves increases when freezing initiates and continues to broaden as the temperature is lowered but there is no abrupt broadening at freezing and subsequent broadening with temperature decrease is almost linear. This suggests that the freezable H_2O in nonacclimated leaves is less structured and less bound and more like pure water, since at the moment of freezing pure H_2O goes abruptly from a very narrow to a very broad bandwidth.

The data suggest that there are significant differences in the status of H_2O and H_2O binding in nonacclimated and acclimated citrus leaves. At above freezing temperatures the broader bandwidth of acclimated leaves could be due to several factors. The association of H_2O molecules with cellular interfaces results in a broadening of the H

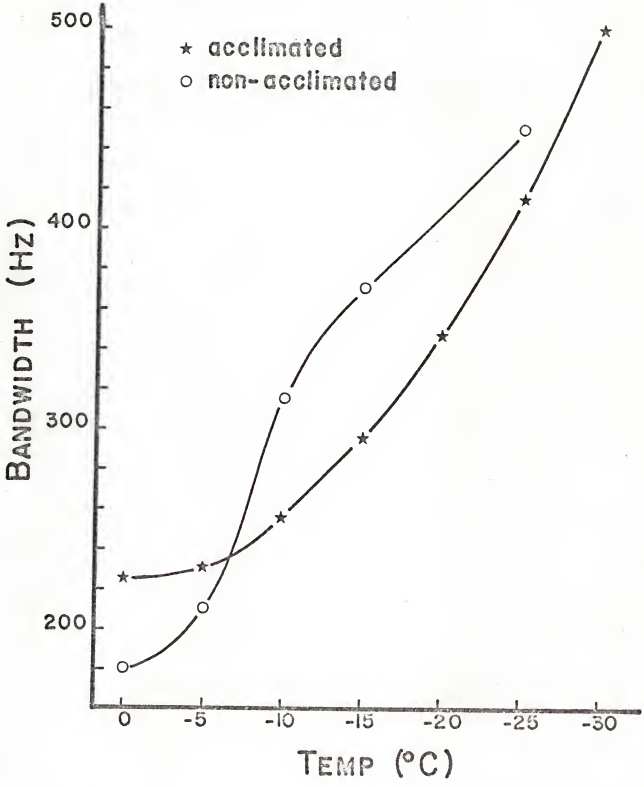

Fig. 4. Temp dependence of H bandwidth of nonacclimated and acclimated deuterated satsuma leaf disks

absorption band and an accompanying reduction of T_2 (33,69). The increase in phospholipids and proteins known to accompany increased cold acclimation suggests that there are increases in cellular interfaces. Increases in aqueous solution viscosity also result in broadening of the H_2O absorption band and a reduction of T_2. The increase in sugars commonly associated with cold acclimation could give rise to a substantial increase in the average viscosity of the tissue solution, resulting in broadening of the H_2O absorption band.

NMR measurements at low temperatures reveal additional differences in the H_2O spectra of nonacclimated and acclimated citrus leaf tissue. At subfreezing temperatures the areas under the absorption bands decrease with reduction in temperature (Fig. 3A). This area is directly related to the amount of nonfrozen bound H_2O in the sample. Comparisons of the relative areas under the absorption bands for nonacclimated and acclimated citrus leaves at the same subfreezing temperatures reveal that acclimated leaves retain a larger fraction of H_2O in the nonfrozen state (Fig. 3A). In both nonacclimated and acclimated leaves, the H resonance signal in H_2O was indistinguishable above the baseline at -50° due to broadening which suggested that all the H_2O had been frozen.

Statistical analysis of the amounts of nonfrozen H_2O using Duncan's multiple range test showed a significant difference between nonacclimated and acclimated leaves when compared at the same subfreezing temperature. The acclimated leaves consistently retained more liquid H_2O. Further, in both nonacclimated and acclimated leaves at subfreezing temperatures, as the temperature was lowered the % of nonfrozen water remaining was significantly less than that at the previous temperature.

The data also showed that when comparing nonacclimated and acclimated leaves, the difference in nonfrozen H_2O at a given subfreezing temperature was not due to the difference in freezing point of the tissue. This was evident when aligning the freezing points to a common reference point and making comparisons of the nonfrozen H_2O at an equal temperature interval from the point of initial freezing (Fig. 3B). Acclimated leaves continually retained significantly more liquid H_2O at all subfreezing temperatures at equal interval from the freezing point, and in both nonacclimated and acclimated leaves, as the temperature interval from the freezing point increased, the % of nonfrozen H_2O remaining was significantly less than that at the previous interval.

These data agree with published findings of other researchers in many ways. These observations indicate that acclimated citrus leaves freeze at a lower temperature than nonacclimated leaves and that the tissue consists, at least in part, of bound H_2O; i.e. H_2O that has restricted motional freedom and is not available for exchange with D_2O. Acclimated citrus leaves after freezing retain a larger portion of bound H_2O as nonfrozen water than do nonacclimated leaves, and additional water is frozen when the temperature is lowered. Further, the larger amount of liquid H_2O remaining at subfreezing temperatures in acclimated leaves is not accounted for by the lower initial freezing temperature. The curve of nonfrozen H_2O in acclimated leaves is different from that of nonacclimated leaves and not just displaced by the temperature difference in freezing.

NMR Deuterium Measurements

The Effect of Freezing on the Spectrum of Liquid D_2O

The NMR spectrum of D in D_2O disappeared at initial freezing in citrus leaf tissue (Fig. 5). Observation of the D spectrum during freezing showed a continual broading with time until a point was reached when no D signal was distinguishable above the baseline. This point coincided with the point on the temperature scale at which all the proton water available for freezing at that temperature had phase-changed from liquid H_2O into ice. This loss of D signal from D_2O with the freezing of D_2O into ice was due to extreme broadening and has been reported by other authors (33).

Because of its electric quadrupole moment, the D nucleus might have provided additional useful information if its signal had remained detectable at temperatures below the freezing temperature of the tissue. It seemed probable that NMR sensitivity to crystallinity might be greater with D_2O than with H_2O since the deuterium nucleus has a quadrupole electric moment which the hydrogen nucleus does not. In a liquid, electric field gradients are small because the electric dipoles of water are randomly oriented in space and randomly moving in time so that the net effect of the electric fields is 0. However, in the presence of order or crystallinity, averaging will decrease, and electric field gradients will increase. Therefore, crystallinity will shorten the NMR relaxation times of the deuteron and broaden the line of its steady-state NMR signal.

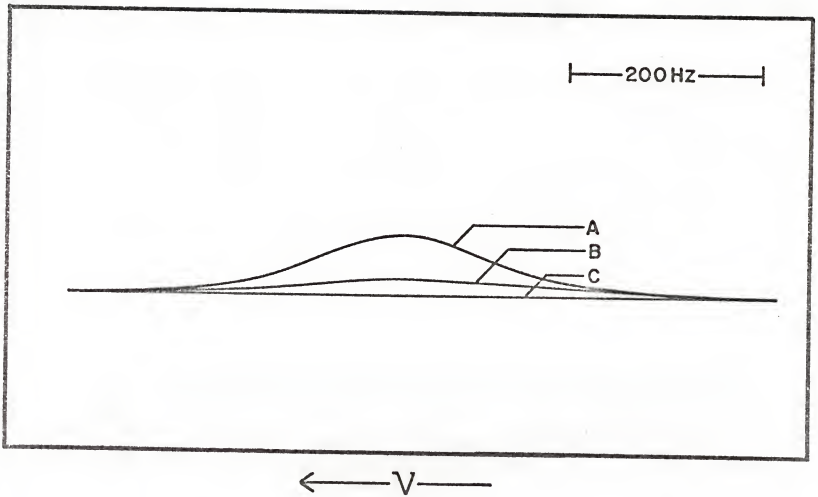

Fig. 5. Typical NMR spectra of D during freezing of nonacclimated samples at -5°C and acclimated samples at -10°C. A) Spectrum before freezing. B) Spectrum after 25 min of freezing. C) Spectrum after 60 min of freezing (spectrum not distinguishable from baseline).

The loss of the NMR deuterium resonance signal at initial freezing suggests that only the free water in the tissue was available for exchange by D_2O, thus eliminating the use of D spectra for studies of bound water characteristics, but allowing D measurements to be used for making measurements of free water.

Standard Curve for D_2O Replacement of H_2O

Since the NMR signals for H_2O and D_2O are not additive to give a quantitative measure of total water, a method was developed that would accurately determine the quantity of H_2O replaced by D_2O in citrus leaf tissue. D_2O was mixed with H_2O in varying proportions and NMR H and D spectra recorded at the temperatures used for citrus leaf samples. A ratio of areas under the absorption bands of H and D was computed and plotted against % D_2O. The ratios of area of H to D are linearly related to the D_2O concentration in H_2O-D_2O mixtures (Fig. 6), and their relationship was used for quantitative analysis for D_2O in H_2O. The NMR spectrum peak height of D in D_2O has previously been used for the quantitative analysis of H_2O-D_2O mixtures (33). However, animal muscle tissue which completely filled the volume in the NMR sample tube was used in these determinations. When using plant leaf material, air space within and between leaves preventsuniform packing of the samples and results in unequal water volume being measured in each sample. Since the actual zone of a sample contributing to the resonance signal cannot be defined, it is difficult to make correction for unequal packing. However, excess leaf material was used in these samples thus assuring

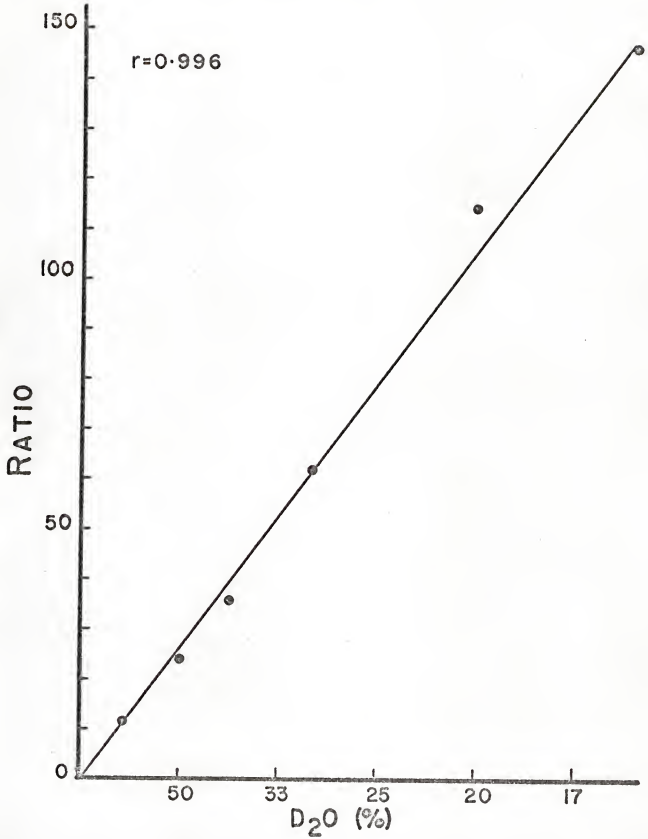

Fig. 6. Standard curve for quantitative analysis of D_2O replacement of H_2O in satsuma leaf disks. % D_2O is % D_2O in H_2O-D_2O mixtures. Ratio = NMR H area/D area.

that samples exceeded the sensitive zone of the receiver coils of the NMR spectrometer.

This standard curve for determining the quantitative exchange of H_2O with D_2O based on the ratio of NMR spectrum from H and D has not been previously reported. This standard curve has a high correlation coefficient and eliminated the need to make assumptions about packing uniformity between samples.

Measurement of Free Water in Citrus Leaf Tissue Using D_2O

The method used for deuteration of citrus leaf disks in this research should expose all intracellular and intercellular tissue water to replacement by D_2O. Evidence has been presented (Fig. 5) that D_2O readily exchanges with only the free water in citrus leaf tissue. Any tissue H_2O not available for exchange with D_2O was considered to be in some way bound. A standard curve for evaluating D_2O in leaf tissue has been developed (Fig. 6). Determinations of free water in citrus leaf disks were made by comparing NMR spectra of H and D to the standard curve.

When citrus leaf disks were floated for 3 hr in 99.7% D_2O, a mean of 69% of the total water was replaced by D_2O in nonacclimated and acclimated tissues (Table 3). The quantity of H_2O replaced by D_2O ranged from 65% to 77% in nonacclimated leaf tissue, and from 61% to 77% in acclimated leaf tissue. However, this deuterated portion did not constitute the total amount of water that froze at initial freezing (Fig. 7). A greater % of the total water in the tissue was lost to ice formation in nonacclimated than acclimated tissue. In nonacclimated tissue 92.3%

Table 3. % H_2O replaced by D_2O in leaf disks from nonacclimated and acclimated satsuma mandarin plants floated in 99.7% D_2O for 3 hr.

Sample[z]	D_2O (%)	
	Nonacclimated	Acclimated
1	77.0	73,3
2	74.1	69.7
3	67.9	73.0
4	69.1	71.9
5	66.2	61.0
6	67.2	63.0
7	66.4	67.3
8	73.0	68.7
9	72.0	64.1
10	65.0	70.4
11	65.5	77.0
12	65.4	72.0
Means	69.1	69.3

[z]Each sample included leaves from 2 experimental plants.

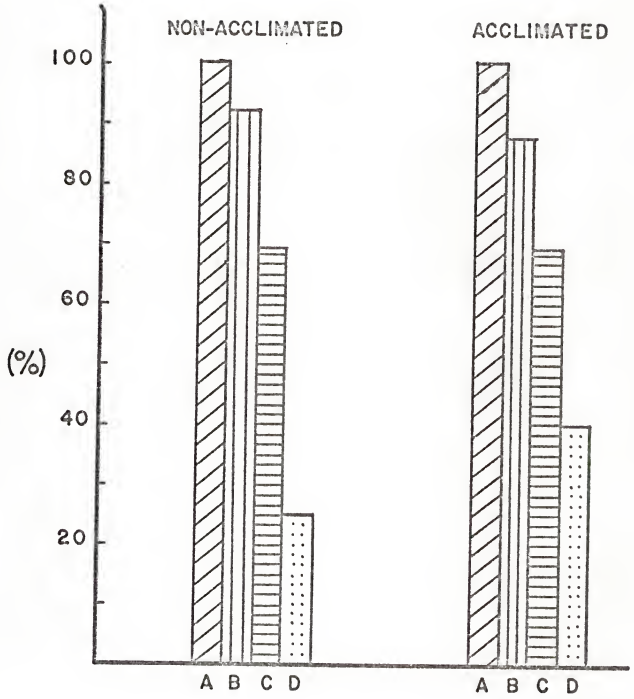

Fig. 7. Water distribution in nonacclimated and acclimated deuterated satsuma leaf disks. A) % total water ($H_2O + D_2O$). B) % total water frozen at initial freezing. C) Total water replaced by D_2O. D) Bound water remaining liquid at initial freezing (as % H_2O not replaced by D_2O).

of the total water froze, and in acclimated tissue 87.9% of the total water froze. This research showed that 7.7% of the total water in non-acclimated citrus tissue remained in the liquid state after initial ice crystal formation due to freezing.

Both nonacclimated and acclimated tissues contained 69% of the total water content as free water, and 31% as bound water as determined by D_2O exchange. The important change in tissue water as a result of acclimation is the smaller quantity of nonexchangeable H_2O which crystallizes at initial freezing. A larger quantity of loosely bound water forms ice at initial freezing in nonacclimated citrus leaf tissue, and a larger quantity of more tightly bound water remains liquid in acclimated tissue.

Conclusions drawn from early research indicate that nonacclimated plant tissue contains a larger portion of its total water as free water and that bound water increases from about 10% to 55% of the total leaf water content during cold acclimation (229). Data reported here did not support those conclusions. This research indicates that the free water in citrus leaves remains relatively constant during cold acclimation, and that the added degree of cold tolerance following acclimation results from increases in the strength of the binding between water molecules during cold acclimation.

The use of NMR to study plant water relations may have made it possible to observe more closely the free and bound water fractions not detectable with other methods. Based on observations of these data, it seems reasonable that a new dimension in the study of free and bound plant water might be realized with future use of NMR.

Experiments with Plant Uptake of D_2O to Establish a Method for Deuterating Citrus Leaves

When excised leaves with attached petioles immersed in 99.7% D_2O were positioned upright in beakers under artificial light, transpiration occurred but at a reduced rate similar to transpiration rates of attached leaves observed at sunset. Transpiration of these excised leaves completely stopped within 24 hr, and the leaves appeared bleached after 72 hr. Terminal stem sections with 4 to 6 leaves attached responded similarly under these conditions. Intact plants grown in glass beads watered with a 99.7% D_2O solution ceased to transpire within 24 hr after being repotted from soil into the glass beads. When the NMR spectra of leaves from these experiments were examined for D, none contained sufficient D for an observable resonance signal.

A more extensive effort was made to obtain deuterated sample material by growing intact plants in solution culture containing 33%, 50%, 70%, and 99.7% D_2O also yielded unsatisfactory results. NMR spectra of H in H_2O and D in D_2O were observed for each plant after 3, 4, 7, 14, and 21 days in solution culture. Plants in 99.7% D_2O ceased transpiration within 48 hr and the root systems produced a putrefying odor associated with decaying tissue. Other plants grown in 99.7% D_2O wilted after 48 hr, followed by leaf abscission. However, a weak barely distinguishable D resonance signal was observed. It was very weak and was not found in all plants in the 99.7% D_2O solution. Plants in 50% and 70% D_2O solutions almost completely stopped transpiration within 7 days. No detectable D resonance signal was observed from leaf samples of these plants. The plants in 33% D_2O were still living after 28 days but transpiration was

greatly reduced. These plants were observed and sampled for an additional 28 days but no NMR D resonance signal was ever observed from these samples. New growth flushes on these plants had shortened internodes and small leaves, but were otherwise normal.

The H resonance signal in these samples was 20% to 25% less than the control in 100% H_2O. The % moisture content of the leaves, determined gravimetrically, also decreased up to 10% when the plants were placed in H_2O-D_2O solutions. The higher the D_2O concentration, the less % moisture content of the leaves. Thus, the decreased H resonance signal could be partially attributed to less total water content, although the % moisture decrease in leaves was less than half that of signal lost. Unequal quantity of sample material also contributed to signal variability. In addition there exists a possibility that there was a selection of H_2O over D_2O by the tissues in the root. This possibility has been suggested in the literature (99).

Most plants recovered after being repotted in soil following their exposure to solution cultures containing 70% or less D_2O. The plants exposed to 99.7% D_2O never recovered. Plants in all other D_2O solution concentrations regained normal growth in succeeding growth flushes.

It was concluded that deuterating citrus leaves by either absorption through the roots or uptake by petiole was not possible. Floating leaf disks in 99.7% D_2O solution gave satisfactory results. This method of floating citrus leaf disks in 99.7% D_2O overcame several obstacles in deuterating citrus leaves. It was possible to get D_2O into the tissue, and with sufficient quantity to exchange with H_2O. The

deuterated material gave a sufficiently detectable D resonance signal and the stacking characteristic of disks facilitated packing sufficient mass of leaf tissue into the NMR sample tube near the sensitive region of the receiver coils.

The time necessary for max deuteration of the leaf disks was determined by measuring change in area under the D resonance absorption peak over time. This experiment showed that most of the D_2O exchange with H_2O occurred within 1 hr, that max deuteration was established within 3 hr and did not change up to 24 hr (Fig. 8). The standard curve for determining D_2O replacement of tissue H_2O (Fig. 6) was used in calculating the values in Fig. 8.

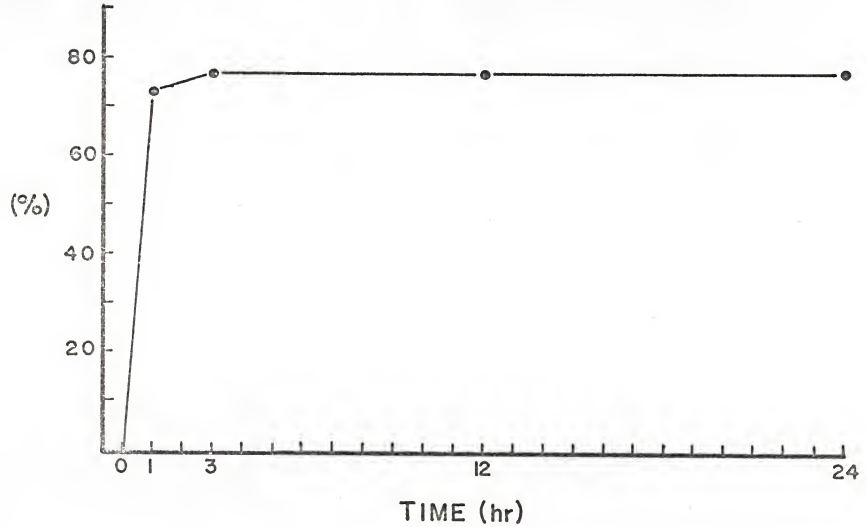

Fig. 8. Exchange (%) of H_2O by D_2O in satsuma mandarin leaf disks at different time intervals.

SUMMARY AND CONCLUSIONS

NMR was used to study the structural characteristics of the non-frozen water in deuterated citrus leaf tissue from nonacclimated and acclimated satsuma plants. Acclimation was induced naturally and in a temperature-controlled chamber. Change in plant hardiness was monitored using the leaf-freezing-point (LFP) method. Temperatures of LFP were in good agreement with temperatures found to initiate freezing in samples observed in the NMR spectrometer.

During freezing, nonacclimated leaves showed an abrupt broadening of the NMR H_2O spectrum as the sample temperature was lowered. This indicates a larger fraction of bound water is subject to freezing at the initial freezing point of the tissue. Acclimated leaves showed a progressively broadening spectrum as the temperature was lowered, but not the abrupt broadening at initial freezing observed in nonacclimated leaves. These results suggest that a significant change in water binding occurs in citrus leaves during cold acclimation. The important change occurring during acclimation seems to be a change in the binding strength between molecules of the water fraction and other cellular constituents. This water has been previously described as the bound water fraction of the tissue water.

At initial freezing and at subfreezing temperatures, acclimated leaves retain a considerably larger % of their total water and bound water in the unfrozen state. At the initial freezing temperature of deuterated tissue all D resonance signal was lost in samples from both nonacclimated and acclimated plants, indicating that all of D was incorporated into the free water and crystallized in the initial formation of ice.

A standard curve for the replacement of tissue water by D_2O was established. A linear relationship between ratios of area under the absorption bands of H and D in plant tissues and ratios based on the D_2O concentration in prepared H_2O-D_2O mixtures was determined. This standard curve made it possible to measure the % H_2O replaced by D_2O in plant material, a measurement which previously has not been considered possible due to the nonuniform packing characteristics of plant materials in the NMR sample tube.

The % of H_2O replaced by D_2O was the same for nonacclimated and acclimated leaves, averaging 69% of the total leaf water content. The quantity of leaf water replaced by D_2O was considered to be the free water because of its ability to readily exchange with D_2O. The remaining leaf water (H_2O) was bound in some manner that prevented its exchange with available D_2O.

A partial loss in the H resonance signal was also observed at initial freezing indicating some of the loosely bound water was forming ice. In acclimated leaves there was a smaller quantity of loosely bound water susceptible to ice formation at initial freezing, and a larger quantity of nonfrozen water remained at subsequent subfreezing temperatures.

Citrus leaf tissue can be deuterated by floating leaf disks in D_2O solutions. Following deuteration by this method, a measurable NMR signal of H in H_2O and D in D_2O can be observed, thus making possible further elucidation of the structural nature of water in citrus leaves. Attempts to deuterate leaves using the natural phenomenon of transpiration of explants and of intact plants grown in solution culture were unsuccessful.

During preliminary tests to determine a satisfactory method for deuterating citrus leaves, certain detrimental effects of D_2O on plants were observed. Transpiration completely stopped within 24 hr after placing leaf petioles in a beaker of 99.7% D_2O under artificial lights and when intact plants were grown in solution culture at this concentration. Plants in 99.7% D_2O solution culture suffered root death. Plants in 33% D_2O solution culture remained alive but showed reduced growth characteristics of short internodes and small leaves. The failure to observe an NMR D signal in leaves from these plants suggests the existence of a mechanism in the roots which not only could distinguish between H and D but could selectively accept one over the other.

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